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Applicant: Gardner et al.

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Examiner: Swope, Sheridan

Title: *Foreign PAS Ligands Regulate PAS Domain Function*

CERTIFICATE OF TRANSMISSION
I hereby certify that this corr is being transmitted by facsimile to the Comm for Patents 571-273-8300 on October 12, 2005.
Signed _____
Richard Aron Osman

RESPONSE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

Thank you for the Action dated Jul 14, 2005; please enter these amendments:

Please amend the title as follows:

~~Foreign PAS Ligands Regulate PAS Domain Function~~ Methods of Regulating PAS Domain Function with Foreign PAS Ligands

Please amend the paragraph bridging p.3 and p.4 as follows:

Figure 3. A) Chemical shift changes plotted against residue number (left) and map of shifting residues with Dd > 0.1ppm (top 20%) on the ribbon diagram of the theoretical model of NPAS2 PAS A (right) for [protein] = 0.25mM and [ligand] = 0.5 mM. The secondary structure elements are shown on top of the bar chart for reference. B) Chemical shift changes plotted against residue number (left) and map of shifting residues with Dd > 0.1ppm (top 20%) on the ribbon diagram of the theoretical model of NPAS2 PAS B (right) for [protein] = 0.25mM and [ligand] = 0.5 mM. Minimum chemical shift changes observed when 0.4 mM of HIF-2 PAS B were mixed with 0.5 mM of compound KG-721.

Please amend the paragraph at p.3, lines 13-20 as follows:

In one aspect of the invention, we show that foreign ligands can be introduced into the hydrophobic core regions of PAS domains even (a) where the PAS domain does not require a core-bound ligand for formation or function; (b) the PAS domain is fully folded in its native state; c) where there is no NMR-apparent a priori formed core cavity to accommodate such a ligand; and/or (d) wherein the PAS domain is unassociated with any predetermined ligand-dependent heterologous chaperone protein. In contrast, AHR PAS-B binds both HSP90, a common chaperone of unfolded proteins, and ligand, and the AHR PAS-B domain is unfolded without ligand (e.g. Kikuchi, et al., 2003, J Biochem 134, 83-90).

Please amend the paragraph at p.14, lines 20-25 as follows:

To validate our chemical library as a discovery tool, we conducted two control NMR-screens against proteins known to bind ligands and previously studied by NMR. The first control target was the immunophilin FK-506 binding protein (Michnick et al., 1991; Rosen et al., 1991). Over the past few years, this protein has been the target of NMR-based screens from which several ligands have been identified and the ligand-binding region has been characterized (Hajduk et al., 1997b; Shuker et al., 1996).

Please amend the paragraph bridging p.14 and p.15 as follows:

A total of 8 hits with binding affinities better than 1 mM were found during our 1H/15N-HSQC-based NMR screen (Figure 1). The map of residues with chemical shift changes ( ) > 0.075 ppm, figure 2A (right), shows that compound KG-190 interacts with FKBP in the same region where FK-506 (shown in magenta on the left) is bound in the crystal structure of the FKBP/FK-506 complex (Van Duyne et al., 1991). Using one-dimensional NMR methods it has previously been shown (Hajduk et al., 1997a) that a compound related to KG-190, 2-phenylimidazole, selectively binds FKBP. Other hits from this screen interacted with the protein in a similar fashion but with lower affinities (scheme 1A), demonstrating the ability of our library to identify a relevant binding site.

Please amend the paragraph bridging p.15 and p.16 as follows:

The first PAS domain protein for which a ligand binding site discovery was made using this library is the PAS A domain of PAS Kinase. The kinase domain of this protein, involved in regulation of sugar metabolism and translation (Rutter et al., ~~2001a~~ 2002), is partially inhibited by direct interaction with its N-terminal PAS domain. We have recently reported that a series of diphenylmethanes, found during a NMR screen (Amezcua et al., 2002), selectively bind at the same site where heme and flavin mononucleotide (FMN) are localized in the crystal structures of FixL (Gong et al., 1998; Miyatake et al., 2000) and Phy3 (Crosson & Moffat, 2001) respectively. The ~~later~~ latter two proteins are also PAS-containing kinases regulated by their PAS domains. This discovery, together with our mutational and biochemical studies, informs the mode of kinase regulation by the PAS domain.

Please amend the paragraph at p.20, lines 2-16 as follows:

NMR Experiments. All NMR experiments were recorded on a Varian Unity Inova spectrometer operating at a proton frequency of 500 MHz, equipped with a SMS autosampler (Varian, Inc.), and a 50-position sample tray. The data was processed with NMRPipe (Delaglio et al., 1995) and analyzed with NMRView (Johnson, 1994). A typical 2D $^{15}N/^{1}H$-HSQC experiment was recorded on samples containing 0.22-0.25mM of U-^{15}N-labeled protein in the appropriate buffer containing 10% D_2O. For the primary screen, each protein sample was mixed with 5 compounds at a final concentration of 0.5 mM each. Similar studies have used higher compound concentrations and larger number of compounds per sample, *e.g.* 1 mM and up to 100

compounds (Hajduk et al., 1999; Hajduk, 1997a,b), however, 0.5 mM/compound and 5 compounds/mixture were a good compromise between the number of observed hits and the amount of protein used for the following steps. Identification of hits was easily accomplished through an in-house module written for NMRView that uses the minimum chemical shift method (Farmer et al., 1996) to rank spectra ($Dd = [dH^2+(dN*0.1)^2]^{1/2}$). Deconvolution of hits was done by adding the individual components of the mix (0.5 mM) to a protein sample (0.25 mM) and ranking the spectra in a similar fashion as described above.

Please amend the paragraph at p.23, lines 17-19 as follows:

Hajduk, P. J., Olejniczak, E. T. & Fesik, S. W. (1997a). One-Dimensional Relaxation- and Diffusion-Edited NMR Methods for Screening Compounds That Bind to Macromolecules. J. Am. Chem. Soc. 119, 12257-12261.

Please amend the paragraph at p.23, lines 20-24 as follows:

Hajduk, P. J., Sheppard, G., Nettesheim, D.G., Olejniczak, E.T., Shuker, S.B., Meadows, R.P., Steinman, D.H., Carrera, G.M., Marcotte, P. A., Jr., Severin, J., Walter, K., Smith, H., Simmer, R., Holzman, T.F., Morgan, D.W., Davidsen, S.K., Summers, J.B., & Fesik, S.W. (1997b). Discovery of Potent Nonpeptide Inhibitors of Stromelysin Using SAR by NMR. J. Am. Chem. Soc. 119, 5818-5827.

Immediately after p.24, line 30, please insert a new paragraph as follows:

Rutter J, Probst BL, McKnight SL (2002) Coordinate regulation of sugar flux and translation by PAS kinase. Cell. 111(1):17-28.

Please amend the Abstract as follows, deleting its first paragraph:

~~Specific binding of a foreign core ligand to a PAS domain, wherein the PAS domain is predetermined, prefolded in its native state, and comprises a hydrophobic core that has no NMR-apparent a priori formed ligand cavity, is determined by (a) detecting a first NMR spectrum of the PAS domain in the presence of a foreign ligand; and (b) comparing the first NMR spectrum with a second NMR spectrum of the PAS domain in the absence of the ligand to infer the presence the ligand specifically bound within the hydrophobic core of the PAS domain.~~

A functional surface binding specificity of a PAS domain, wherein the PAS domain is predetermined, prefolded in its native state, and comprises a hydrophobic core that has no NMR-apparent a priori formed ligand cavity, is changed by (a) introducing into the hydrophobic core of the PAS domain a foreign ligand of the PAS domain; and (b) detecting a change in the functional surface binding specificity of the PAS domain.